nature portfolio

Peer Review File

A hammerhead ribozyme selects mechanically stable conformations for catalysis against viral RNA

Corresponding Author: Professor Zhongbo Yu

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors performed exciting experiments to demonstrate an interesting story about the conformational changes of Ribozyme using a technically appropriate approach.

Reviewer #2

(Remarks to the Author)
The manuscript by Lu et al. describes single molecule pulling experiments on a hammerhead ribozyme to understand its conformational states. The authors used optical tweezers to mechanically unfold the ribozyme and analyzed the force versus distance/extension data to separate conformers they named alpha, beta1-2 and gamma1-2. They found that adding magnesium ions shifts beta1 into beta 2 state, and gamma conformers disappear after adding substrate RNA. Combined with molecular dynamics simulations they previously published, these experiments confirm the multi-state nature of hammerhead ribozymes, which was previously known.

The main problem with this work is the lack of rigor and control experiments.
- The authors should add appropriate negative and positive controls to their pulling experiments.
- How many individual traces were analyzed in each case, how was convergence confirmed, and what is the confidence in the conformer distributions? Can you calculate the relative stability between the conformational states? (The authors mention “free energy difference between beta conformations” in Methods section line 361, but there is nothing reported in the manuscript. I am guessing this was lifted from their previous MD manuscript.)
- Does a specific molecule when unfolded, fold back to its original state?
- The Data Analysis section in Methods needs to be expanded to describe more details.
- There is only a single construct that was studied: Is there any effect of changing the sequences in Table S1? What happens with inactive (mutant) ribozyme after adding RNA? What is the effect of ions other than magnesium?

If the experiments upon adding RNA reports on the “aftermath of the cleavage reaction” (line 263) these observations are not relevant (or do not provide any information on) the mode of binding. Thus, the claim of “induced fit mechanism” does not make sense. The substrate RNA may be preferentially binding to the alpha and/or beta state and thus shifting the equilibrium away from gamma (without “inducing” any change).
-Related to this: If the gamma state is self-inhibited and interaction with RNA results in shift in conformer distribution into the active beta state, this implies the ribozyme should become more “active” – can this be confirmed by the cleavage assay shown in the gel in Figure S2?

It's not clear what is new with the MD simulations results, which seem to be already published. It’s also not clear how well the conformers seen in MD correlate with the experiments:
- Is the conformational distribution similar? Can you assign alpha, beta1-2 and gamma1-2 states from MD? Which conformer is the most relevant for biological activity and ribozyme-based design?
- The alpha state seems to be mostly unfolded. Can this be due to construct design or experimental conditions, or is it a common and required state for all hammerhead ribozymes?

It would be helpful to include a structural (or MD) figure showing the catalysis/core and accessibility in relevant states, which is important to understand the self-inhibited and induced-fit claim by the authors.

Reviewer #3

(Remarks to the Author)
Man Lu et al used single-molecule magnetic tweezers to explore the unfolding and folding landscapes of a hammerhead ribozyme. Their results revealed the presence of multiple RNA intermediates along the folding and unfolding pathways, shedding light on the mechanism of conformational selection and induced-fit. The mechanical conformations were further validated through MD simulations. Understanding the mechanism of conformational selection and induced-fit is crucial in the field of enzymatic catalysis, and experimental evidence plays a pivotal role in this.

This study provides quantitative experimental evidence at the single-molecule level, supporting and advancing the biological theory of conformational selection and induced-fit. Therefore, I highly recommend the publication of this study in Communications Biology, pending the resolution of the following concerns.

1. Figure 1 should show a schematic drawing of the hammerhead ribozyme that illustrates all its domains and mechanistic bonds, rather than the simple core domain and helices. This will aid readers in better understanding the ribozyme's structure.

2. In Figure 2C, the authors have identified two mechanical populations in the green panel, whereas in Figure 3C, only one population is marked. However, upon closer examination, it can be observed that the green panel of Figure 3C also exhibits two populations, albeit with a lower probability of one being omitted. It would be more equitable to discuss the fluctuation in probabilities rather than completely omitting one intermediate.

3. To enhance reader-friendliness, it is suggested that Figure 4b and 4d include marks indicating the disappearance of the gamma population, thus illustrating the significant conformational selection that occurs due to the ribozyme's substrate.

4. To further enhance our understanding of the ribozyme's unfolding pathway, the inclusion of a series of MD snapshots would be valuable.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This form is now publishable.

Reviewer #3

(Remarks to the Author)
My previous comments have been well revised and appropriately resolved. With no further concerns, thus I highly recommend the publication of this work in Communications Biology.

Reviewer #1:

The authors performed exciting experiments to demonstrate an interesting story about the conformational changes of Ribozyme using a technically appropriate approach.

Response from the authors:

We sincerely appreciate the Reviewer's encouraging comments.

Review comments

1. From line number 138 to 141, the CUGA loop in Fig1a, is followed by helix 3. It might need to be changed that "CUGA loop following the helix I" to be "helix 3".

Response from the authors:

We appreciate the Reviewer's insightful comment. We acknowledge that the text description does not match the illustration in Figure 1a. Instead of changing the text, we have updated the scheme in Figure 1a accordingly. As a result, the text description is now consistent with the illustration in Figure 1a.

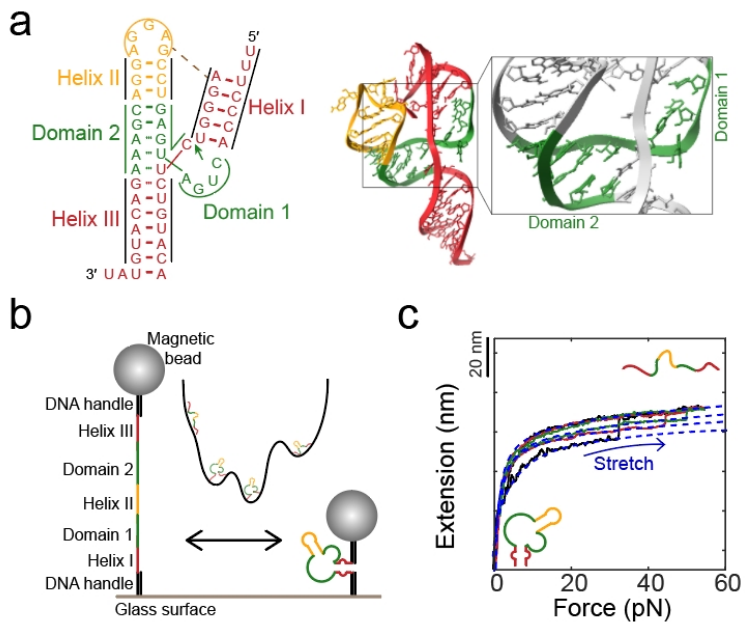

Figure 1. The structures of a mini hammerhead ribozyme targeting the genomic RNA sequence of SARS-CoV-2 and the experimental setup for single-molecule studies

(a) Schematic representation of the mini hammerhead ribozyme highlighting helices I-III and domains 1-2 (left), alongside the 3D structure obtained from molecular dynamics simulations (right). The green arrow marks the cleavage site.

2. The authors need to clarify how data are classified in Fig 2b among three data. The reader can have confusion whether the three categories made by three Gaussain functions. Also, whether gamma and beta sets were identified by MT extension need to be clarified.

Response from the authors:

We appreciate the Reviewer's questions regarding the classification of data in Figure 2b.

To address the first point, the data in Figure 2b are classified into three categories based on their distribution, which was modeled using three Gaussian functions. We understand that this might cause some confusion, so we have added a clarification in the Statistics and reproducibility section of the Methods to explicitly state that the three categories are indeed the result of fitting the data with three Gaussian functions (as detailed below).

Regarding the second point, the identification of the gamma and beta sets was based on Gaussian populations derived from changes in extension, rather than directly from individual force-extension curves measured by MT. We have clarified this in the Statistics and reproducibility section of the Methods to ensure that readers understand the identification process (as also detailed below).

Revised Statistics and reproducibility Section in the Methods:

"We measured the changes in extension of cooperative unfolding events in force-extension curves. The distribution of changes in extension was modeled using Gaussian functions. The number of Gaussian functions used for modeling was determined by statistical analysis. Typically, two or three Gaussian functions have been used. The conformations were thus identified by the Gaussian populations, i.e., α, β, and γ sets."

3. In Fig. S1, the main text seems to be trying to say that there are two gamma states because, looking at the gamma set, helix3 appears to interact with helix2 in one case and helix1 in the other case. However, in Fig. S1 below, it appears that helix2 is classified into two cases where helix2 interacts with helix1 or helix3 than expected. In addition, in order for the largest step of the structure to occur in a structure where DNA is connected and pulled at the ends of helix 1 and 3, the simulation results show that the central core must be opened. However, in the case of the gamma set and beta set, helices 1 and 3 are the same in an open form, and even if the interaction with helix 2 of the gamma set falls under externally applied tension, it is difficult to determine whether it is sufficient to create a step of 20 to 30 nm. However, since this is a simulation result, it is unknown what form the molecules will actually take.

Response from the authors:

We appreciate the Reviewer's insightful comments. We acknowledge the notable differences between the MD simulation results and the single-molecule mechanical unfolding experiments for the mini ribozyme. In the single-molecule unfolding assays, the changes in the extension observed in the γ conformation suggest that helices I and III can either interact directly or both engage with helix II or the core domains. To address this, we conducted additional MD simulations and captured more instances of the γ conformation. These simulations confirm that helices I and III can either interact directly or both engage with the core domains, as reflected in the updated Figure S3b (Figure S1b in the previous version). The revised MD results provide a clearer understanding of these interactions, and the updates have been incorporated into Figure S3. We have also clarified the description of the γ conformation in the main text (line 213), as follows:

"Using all-atom MD simulations, we further revealed that in the mini ribozyme γ conformation, helices I and III can either directly interact or both engage with the core domains (Figure S3)."

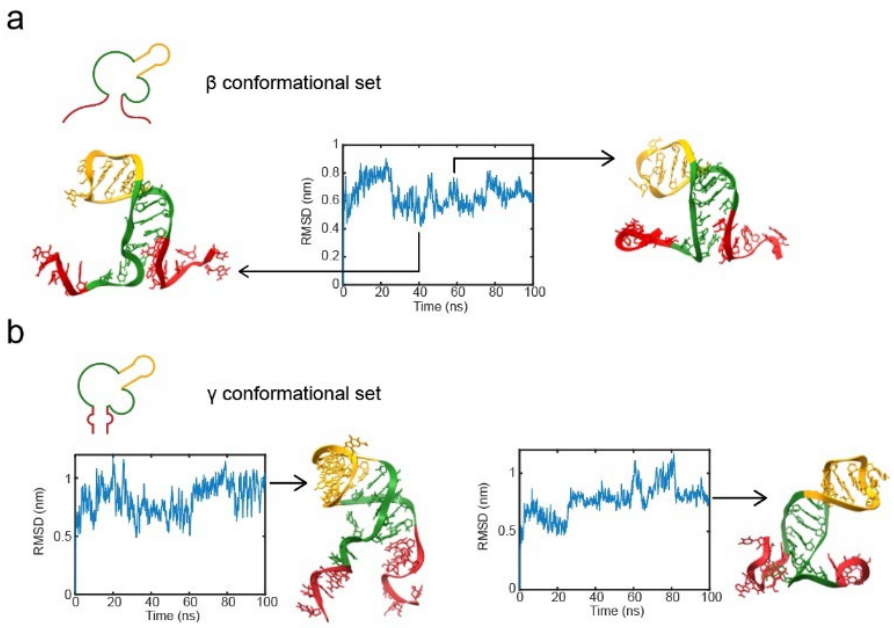

Figure S3. Conformational sets of the mini ribozyme revealed by MD simulations

Snapshots from MD simulations without force loading are shown, with the corresponding times indicated by arrows.

4. It seems clear that the addition of magnesium causes one of the possible states of β set to disappear. However, there are still the following problems: Even if the data set for the extension is classified for each set using Gaussian, if there is a data point that can enter the alpha or beta, how can the data be classified

if the power data contained in the data is not included as a classification criterion for dividing the set? Can we discuss whether a point is alpha or beta?

Response from the authors:

We have identified the α, β, and γ sets of changes in extension based on Gaussian populations. For the corresponding force distributions, we statistically assigned the force data to the α, β, or γ sets based on Gaussian probability on a randomized basis. As a result, we cannot definitively determine whether a specific point belongs to the α or β set; instead, we can provide the probability of a data point belonging to either the α or β set. This approach and its implications have been addressed in the Statistics and reproducibility section of the Methods, as detailed below.

Revised Statistics and reproducibility Section in the Methods:

"For the corresponding force distributions, we statistically assigned the force data to the α, β, or γ sets based on Gaussian probability on a randomized basis."

5. To comment on the last part, it seems clear that gamma set disappears in the experiment with the addition of viral RNA. However, did the viral RNA infiltrate and bind to the hammerhead ribozyme while the DNA was completely condensed? Or did the viral RNA flow while the DNA was unfolded, incubate, condense the hammerhead ribozyme, and then pull it again? There seems to be a need to check if it is a process. In the former case, induced confirmation appears to have been sufficiently induced by viral RNA. In the latter case, the gamma set may have disappeared because the RNA was folded after attachment with the complementary binding site artificially exposed.

Response from the authors:

We appreciate the Reviewer's insightful comments. To address the question regarding the conformation of the DNA-RNA-DNA construct, we performed AFM imaging. The results showed that the two DNA handles, each approximately 200 nm in length, do not condense but rather spread out in a Tris buffer (Figure S2). This behavior can be attributed to the persistence length of DNA, which is about 51 nm, preventing the relatively short DNA handles from condensing and obscuring the ribozyme. Although the ribozyme is positioned between the two DNA handles, it remains sufficiently exposed and accessible to the RNA substrate. Therefore, in our single-molecule experimental setup, the ribozyme's conformation can be modulated by its RNA substrate without significant hindrance.

We have clarified this point at line 153 in the main text as follows:

“Although the ribozyme is positioned between the two DNA handles, it remains sufficiently exposed and accessible to the RNA substrate, as revealed by AFM imaging (Figure S2).”

We detailed the AFM imaging procedure in the Methods section as follows:

“We employed a Multimode 8 AFM instrument manufactured by Bruker (Billerica, USA) and conducted measurements at room temperature (23 °C). The mica surface was prepared by peeling it with tape to ensure flatness and cleanliness. Prior to loading the sample, we mixed the DRD construct with 10 mM $NiCl_2$ in a 40 mM Tris buffer (pH 8.0). We then applied 10 µl of the mixture to the center of the mica surface and allowed it to settle for 3 minutes. Afterward, the sample was gently rinsed with water and dried using nitrogen gas. For scanning and high-resolution imaging, we used an SNL-10 probe, also from Bruker. The scanning parameters were optimized to ensure the best match between the trace and retrace curves. The scan size was gradually increased from 10 nm to 1000 nm to identify the appropriate range, and the XY offset was adjusted after each scan to explore different areas. Finally, AFM images were analyzed using NanoScope software.”

As pointed out in the 6th question by Reviewer 2, we acknowledge that we did not directly observe or measure the induced-fit process. Consequently, we have removed the statement regarding "induced-fit" in this revision. As detailed in our response to the 6th question below, we have developed a new single-molecule setup to directly monitor the ribozyme's digestion of the RNA substrate, and we will report these results in the future, which should provide direct insight into the induced-fit process.

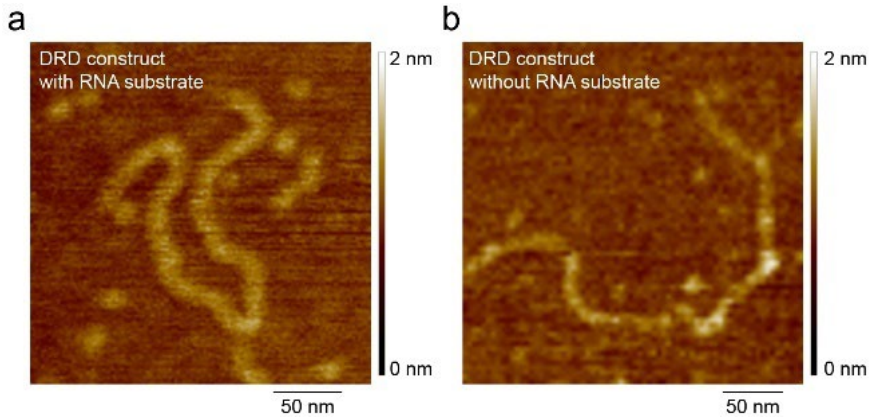

Figure S2. AFM imaging of the DNA-RNA-DNA construct of the mini ribozyme with and without its substrate

6. Lastly, if the headhammer ribozyme acts as a catalyst for viral RNA naturally induced by a stable equilibrium state in the presence of magnesium, it would be a good idea to check whether the viral RNA is decomposed and released in an MT experiment. Is the data provided in the supplementary actually the result of cutting RNA into which a ribozyme was added in a beaded DNA setup? Or did you mix the ribozyme solution used in the experiment in bulk with the viral RNA and then run electrophoresis over time to check for decomposition? The reason why this question is important is that the ribozyme is in a state of force due to external tension. However, if the RNA is not decomposed even though a very small force is applied to the MT after RNA and magnesium are introduced, the specific state discovered through MT This is because these are not states that actually participate in RNA decomposition, and the confirmation effective for the actual catalyst is a third similar confirmation excluding modification by MT.

Response from the authors:

We appreciate the Reviewer's insightful suggestion. To accurately assess whether viral RNA is digested and released in a MT experiment, we believe a redesigned single-molecule mechanical assay would be more suitable. In this proposed design, magnetic tweezers would be used to hold the viral RNA substrate between a magnetic bead and a glass surface, rather than holding the ribozyme as in our current setup. This would allow the ribozyme to freely diffuse in the buffer and interact with the viral RNA substrate. In this new setup, upon ribozyme binding, the single-stranded viral RNA substrate would be expected to adopt a double-stranded helix conformation, leading to a shortening of the contour length, which could be detected by magnetic tweezers. If digestion occurs, the viral RNA would break, causing the magnetic bead to detach and "fly away", which the magnetic tweezers could detect. We have prepared a DNA-viral RNA-DNA construct for this new design and currently are performing single-molecule assays and MD simulations. However, the current viral RNA substrate is only 14 nucleotides long, and the expected change in contour length from single-stranded to double-stranded RNA is approximately 2 nm, which challenges the precision of our single-molecule experiment. It will take considerable time to systematically investigate ribozyme-RNA substrate binding and real-time digestion using this approach, which we cannot complete within this revision. We plan to continue this investigation and report our findings in the future.

Regarding Figure S2 in the previous version, in the ribozyme activity assays presented, there were no magnetic beads involved in the reaction system. The assays were conducted with RNA only, without the DNA handles used in single-molecule assays, meaning the ribozyme was not subjected to any external forces, and its conformations were unaffected by such forces. In the single-molecule mechanical assays, although the ribozyme was attached to a magnetic bead, enzyme digestion occurred during the zero-force

intervals between sequential stretching force-extension assays. Therefore, the active conformation of the ribozyme was not influenced by external forces. The force-extension assays served as a detection method to probe the ribozyme-RNA substrate interactions and had minimal mechanical impact on the digestion process.

We have clarified this point in the caption of Figure S4 by adding the following sentence:
"The mini ribozyme consists of RNA only, without the DNA handles used in single-molecule mechanical experiments."

We hope these clarifications address your concerns and provide a clearer understanding of our experimental design and the implications of the observed results.

Reviewer #2:

The manuscript by Lu et al. describes single molecule pulling experiments on a hammerhead ribozyme to understand its conformational states. The authors used optical tweezers to mechanically unfold the ribozyme and analyzed the force versus distance/extension data to separate conformers they named alpha, beta1-2 and gamma1-2. They found that adding magnesium ions shifts beta1 into beta 2 state, and gamma conformers disappear after adding substrate RNA. Combined with molecular dynamics simulations they previously published, these experiments confirm the multi-state nature of hammerhead ribozymes, which was previously known.

Response from the author:

We are sincerely appreciative of the reviewer's encouraging comments.

1. The main problem with this work is the lack of rigor and control experiments. The authors should add appropriate negative and positive controls to their pulling experiments.

Response from the authors:

We appreciate the Reviewer's constructive comment. In response to this concern, we have conducted additional experiments with both negative and positive controls. Specifically, we tested an inactive ribozyme containing a destructive mutation, as well as a smaller but active ribozyme with a shorter stem II domain compared to the current mini ribozyme. Further details can be found in our 5th response. Additionally, we performed more MD simulations and AFM imaging to provide supporting evidence, as detailed in the 3rd and 5th responses to Reviewer 1, the 7th and 8th responses to Reviewer 2, and the 4th response to Reviewer 3.

2. How many individual traces were analyzed in each case, how was convergence confirmed, and what is the confidence in the conformer distributions? Can you calculate the relative stability between the conformational states? (The authors mention "free energy difference between beta conformations" in Methods section line 361, but there is nothing reported in the manuscript. I am guessing this was lifted from their previous MD manuscript.)

Response from the authors:

We sincerely appreciate the Reviewer's constructive comments and have made the following revisions to address them:

- Number of Traces

We have now included the number of individual traces analyzed in each figure caption. Generally, the number of traces exceeds 50 for each data set, ensuring robust statistical analysis.

- Convergence and confidence of Distributions

To ensure the convergence of each distribution, we have collected total 1619 data points for all the tested conditions, which is considered a sufficiently large sample size for single-molecule assays conducted with super high instrumental precision. The variability inherent in our single-molecule measurements was carefully analyzed, confirming that the distributions are well-defined and exhibit clear shapes, indicative of convergence. The confidence in the conformer distributions has been quantified and is now reported alongside the data. The confidence intervals reflect the degree of certainty in our measurements and provide a clear statistical basis for interpreting the distributions. The statistics for each data set are now provided in Table S2 and noted at line 190 in the main text.

Table S2. Statistics of single-molecule experiments

Data set	N	Mean	Standard deviation	95% confidence interval
Fig 2b	462	13.4 nm	7.2 nm	13.4 ± 0.7 nm
Fig 2c (left)	180	38.6 pN	11.6 pN	38.6 ± 1.7 pN
Fig 2c (middle)	187	35.0 pN	12.2 pN	35.0 ± 1.8 pN
Fig 2c (right)	95	36.8 pN	12.9 pN	36.8 ± 2.6 pN
Fig 3b	339	13.1 nm	6.5 nm	13.1 ± 0.7 nm
Fig 3c (left)	128	38.9 pN	9.8 pN	38.9 ± 1.7 pN
Fig 3c (middle)	159	36.5 pN	13.3 pN	36.5 ± 2.1 pN
Fig 3c (right)	52	37.2 pN	14.3 pN	37.2 ± 4.0 pN
Fig 4b	235	9.4 nm	5.0 nm	9.4 ± 0.7 nm
Fig 4c (left)	99	36.3 pN	11.1 pN	36.3 ± 2.2 pN
Fig 4c (right)	136	34.7 pN	11.6 pN	34.7 ± 2.0 pN
Fig 4d	133	9.2 nm	4.8 nm	9.2 ± 0.8 nm
Fig 4e (left)	78	40.3 pN	10.2 pN	40.3 ± 2.3 pN
Fig 4e (right)	55	38.2 pN	10.9 pN	38.2 ± 3.0 pN
Fig S6b	88	11.3 nm	6.4 nm	11.3 ± 1.4 nm
Fig S6c (left)	52	41.8 pN	10.3 pN	41.8 ± 2.9 pN

Fig S6c (right)	36	37.4 pN	11.5 pN	37.4 ± 3.8 pN
Fig S6d	141	9.9 nm	4.5 nm	9.9 ± 0.8 nm
Fig S6e (left)	111	37.8 pN	12.0 pN	37.8 ± 2.3 pN
Fig S6e (right)	30	37.1 pN	11.6 pN	37.1 ± 4.2 pN
Fig S7b	62	10.6 nm	6.7 nm	10.6 ± 1.7 nm
Fig S7c (left)	38	20.1 pN	6.8 pN	20.1 ± 2.2 pN
Fig S7c (right)	24	40.4 pN	9.1 pN	40.4 ± 3.7 pN
Fig S7d	49	9.6 nm	6.6 nm	9.6 ± 1.7 nm
Fig S7e (left)	25	29.6 pN	9.8 pN	29.6 ± 3.9 pN
Fig S7e (right)	24	39.4 pN	10.8 pN	39.4 ± 4.4 pN
Fig S7f	60	8.6 nm	3.6 nm	8.6 ± 0.9 nm
Fig S7g (left)	27	39.8 pN	8.8 pN	39.8 ± 3.4 pN
Fig S7g (right)	33	41.5 pN	10.5 pN	41.5 ± 3.7 pN
Fig S7h	50	6.2 nm	2.3 nm	6.2 ± 0.6 nm
Fig S7i (left)	29	42.0 pN	8.1 pN	42.0 ± 3.0 pN
Fig S7i (right)	21	38.5 pN	10.0 pN	38.5 ± 4.4 pN

- Relative Stability of Conformational States

Regarding the relative stability of the conformational states, we have now included experimental calculations. The experimental free energies of conformations were estimated using Jarzynski's equation. This method, initially developed by C. Jarzynski and experimentally validated by J. Liphardt et al., allows us to calculate the free energy difference between the mechanical conformations [1,2]. The free energy differences between states are summarized in Table S3 and noted at line 210 in the main text.

Table S3. Stability of conformational states revealed by single-molecule experiments

Data set	ΔG (k_BT) of α state	ΔG (k_BT) of β1 state	ΔG (k_BT) of β2 state	ΔG (k_BT) of γ1 state	ΔG (k_BT) of γ2 state
Fig 2c (left)	-62.87	---	---	---	---
Fig 2c (middle)	---	-73.33	-151.84	---	---
Fig 2c (right)	---	---	---	-140.29	-295.46
Fig 3c (left)	-65.20	---	---	---	---

Fig 3c (middle)	---	---	-127.98	---	---
Fig 3c (right)	---	---	---	-106.73	-260.88
Fig 4c (left)	-53.72	---	---	---	---
Fig 4c (right)	---	---	-91.10	---	---
Fig 4e (left)	-59.15	---	---	---	---
Fig 4e (right)	---	-82.08	-166.28	---	---

We also provided the detailed calculation method, including the equation, in the note of Table S3:

$\exp[-\beta \Delta G(z)] = lim_{N\to\infty} < \exp\,[-\beta w_i(z,r)] >_N$

where $< >_N$ denotes averaging over N work traces, $w_i(z,r)$ represents the work done in the ith trace, and r is the switching rate. The mechanical work $w_i(z,r)$ required to switch the system between positions 0 and z under an external force F is given by:

$$w_i(z,r) = \int_0^z F_i(z',r)dz'$$

where $F_i(z',r)$ is the external force applied at position z' with switching rate r.

3. Does a specific molecule when unfolded, fold back to its original state?

Response from the authors:

We appreciate the Reviewer's insightful comment. Our mechanical unfolding assays were performed on single molecules with sufficiently long intervals between measurements. Specifically, the interval between consecutive unfolding assays is typically longer than 5 minutes, allowing the ribozyme to refold in the absence of any applied force (at zero force). Our results indicate that the ribozyme does not consistently fold back to its original conformation. Instead, it exists in an ensemble of different conformational states, specifically the α, β, and γ conformations. Repeated unfolding experiments on the same molecule show that the ribozyme can refold into multiple distinct conformations rather than returning to its initial state. This variability in folding pathways suggests a dynamic conformational landscape rather than a single, defined folding state.

We have addressed this observation in detail in the Methods section under "Single-molecule Mechanical Assays," emphasizing the experimental conditions that allow for the observation of multiple folding conformations.

"The time interval between two sequential force-extension curves is generally set to be longer than 5 minutes, which ensures that a single molecule can refold in the absence of any applied force, allowing us to observe the range of possible conformations the ribozyme can adopt."

4. The Data Analysis section in Methods needs to be expanded to describe more details.

Response from the authors:

We appreciate the Reviewer's constructive comment and have added the Statistics and reproducibility section to provide a more detailed explanation of our methods. Below are the key additions:

"We measured the changes in extension of cooperative unfolding events in force-extension curves. The distribution of changes in extension was modeled using Gaussian functions. The number of Gaussian functions used for modeling was determined by statistical analysis. Typically, two or three Gaussian functions have been used. The conformations were thus identified by the Gaussian populations, i.e., α, β, and γ sets. For the corresponding force distributions, we statistically assigned the force data to the α, β, or γ sets based on Gaussian probability on a randomized basis. The statistics of single-molecule experiments are summarized in Table S2."

Furthermore, we have provided additional details on the energy calculations in the note for Table S3, where we describe the process of calculating the free energy differences between conformations using the observed force-extension data.

5. There is only a single construct that was studied: Is there any effect of changing the sequences in Table S1? What happens with inactive (mutant) ribozyme after adding RNA? What is the effect of ions other than magnesium?

Response from the authors:

We appreciate the Reviewer's insightful comments. To address the concern regarding the impact of sequence changes on the ribozyme, we designed an additional hammerhead ribozyme with the sequence 5'-UUCCCACUGAUGAGACUGGACGAAAGACAUGUAU. Both this new ribozyme and the original ribozyme target the RNA sequence 5′-ACAUGUCUCUGGGA of SARS-CoV-2. However, the new ribozyme, referred to as the "tiny ribozyme," features a shorter helix II stem and a longer loop compared to the mini ribozyme (Figure S6a).

Using single-molecule mechanical assays in the absence of magnesium and RNA substrate, we observed that the tiny ribozyme exhibits four mechanical conformations with two distinct extension changes (Figure S6b-c). This observation supports our previous finding that ribozymes can adopt conformations with varying mechanical stabilities. In contrast, the mini ribozyme displays five mechanical conformations with three distinct extension changes, likely due to the differences in the helix II stem length, which may influence the adjacent core domains and affect the number of mechanical conformations.

Upon adding 10 mM magnesium, the tiny ribozyme continued to display four mechanical conformations with two extension changes (Figure S6d-e), but there was a notable decrease in the population exhibiting the longer extension change (Figure S6b vs S6d, blue). Similarly, in the mini ribozyme, the population with the longest extension change also decreased (Figure 3b vs. 2b). The longer extension changes observed in mechanical unfolding assays are indicative of long-range domain-domain interactions. The reduced populations suggest that magnesium facilitates further folding of the inner core domains, potentially disrupting these interactions and resulting in fewer conformations involving long-range domain-domain interactions.

Furthermore, for the tiny ribozyme, the two mechanical conformations with short extension changes exhibited different distributions before and after the addition of magnesium. Post-magnesium addition, the population of the weaker mechanical conformation decreased, resulting in a relative increase in the stronger mechanical conformation (Figure S6c vs S6e, arrows in the right panels). This observation is consistent with findings for the mini ribozyme, suggesting that magnesium ions selectively stabilize the mechanically stronger conformations, thereby shifting the equilibrium toward these more stable states.

These results demonstrate the influence of sequence variations on the folding and mechanical properties of ribozymes, highlighting the role of specific structural elements in ribozyme function. We have addressed this point in the main text at line 279 and incorporated the findings in Figure S6 of the Supplementary Information.

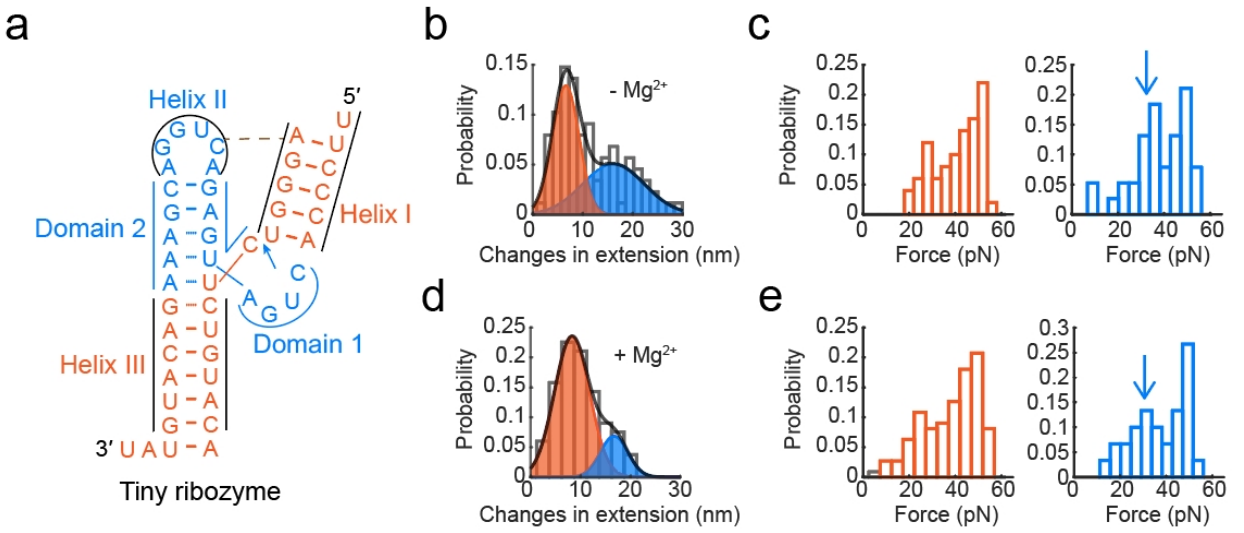

Figure S6. Single-molecule mechanical assays of tiny ribozyme

Blue arrows indicate the mechanical states that respond to magnesium ions. Statistical data are provided in Table S2.

To address the concern regarding the inactive mutant, we designed an inactive mini ribozyme with the sequence 5′-UUUCCCACUGAU**dG**AGUCCGAGGAGGACGAAAGACAUGUAC (Figure S7a and Table S1). The inactive ribozyme cannot digest RNA substrate due to the presence of a deoxynucleotide at the catalytic center, indicated by the bold dG in the sequence. However, the inactive ribozyme retains its ability to recognize and bind the RNA substrate via the helix I and III domains, which are complementary to the RNA substrate.

Single-molecule mechanical assays revealed the presence of α and β conformations but no γ conformations, irrespective of the presence of magnesium (Figure S7b-e). This suggests that the deoxynucleotide dG in the core domain destabilizes the γ conformations, reinforcing our previous findings that the γ conformations are based on tertiary interactions involving the core domains, as evidenced by both our single-molecule assays and MD simulations. Additionally, we observed only one mechanical state of the β conformation in the inactive mini ribozyme, in contrast to the two mechanical states of the β conformation in the active mini ribozyme, suggesting that the mutated guanosine affects interactions with magnesium ions. In the presence of the RNA substrate, the β conformations of the inactive mini ribozyme become shorter, suggesting that the deoxynucleotide dG partially disrupts the core domains (Figure S7f-i).

We have addressed these points in the main text at line 345 and incorporated the findings in Figure S7 of the Supplementary Information.

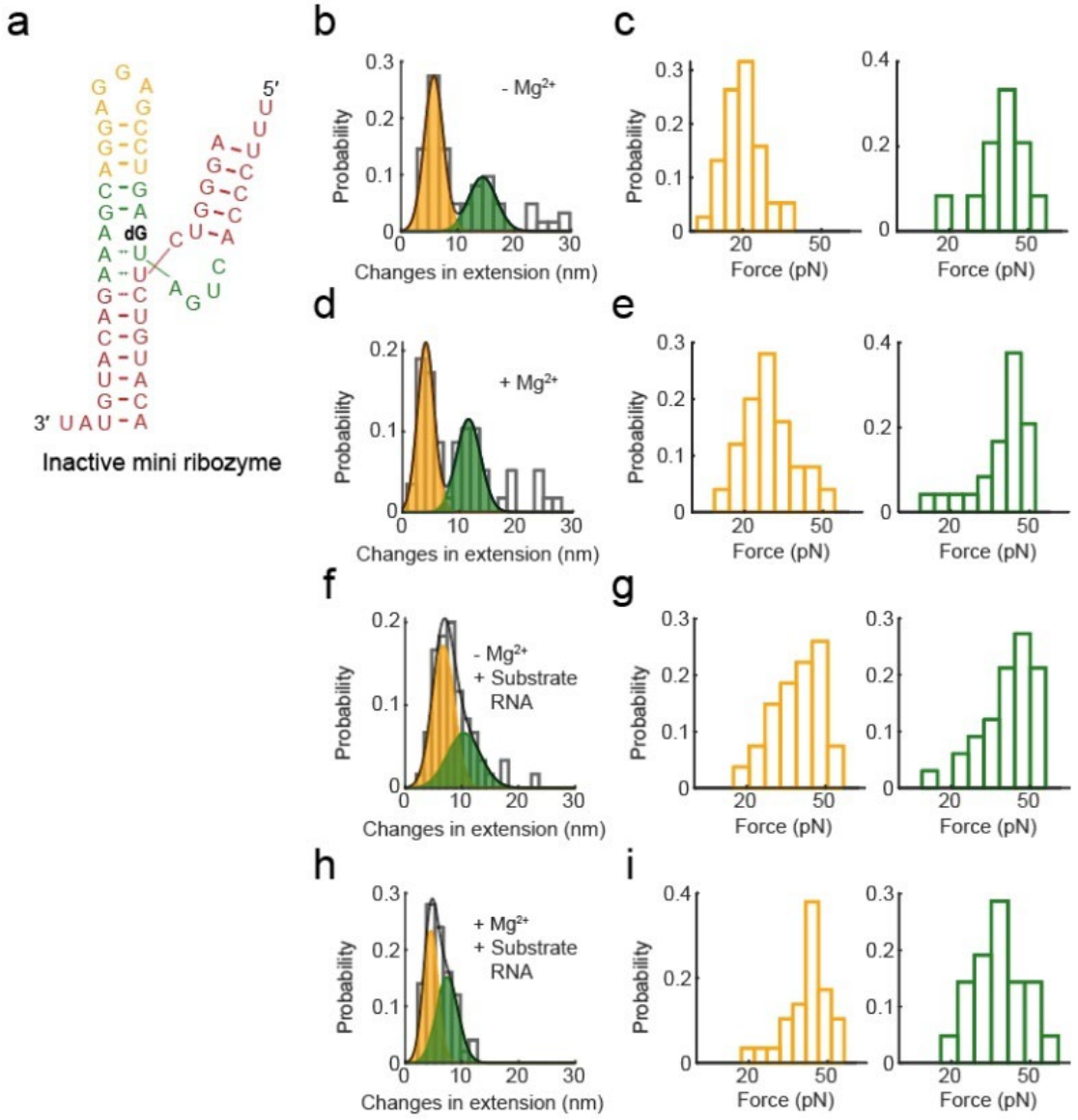

Figure S7. Single-molecule mechanical assays of inactive mini ribozyme

Statistical data are provided in Table S2.

Divalent metal ions play a crucial role in the proper folding and catalysis of hammerhead ribozymes, and both their structures and functions are highly sensitive to these cations[3]. Magnesium ions, in particular, are widely considered as the physiologically relevant ion that can activate the catalytic activity of hammerhead ribozymes[4,5]. To address the reviewer's concern about the ion effect, we conducted additional MD simulations under both calcium and magnesium ion conditions. Our results, observed over

a 100 ns time scale, indicate that the mini ribozyme remains relatively rigid at the helix II domain (Figure S5). The conformational dynamics primarily occur within the core domains, especially at the catalytic bases and in the interactions between helix I and helix III. Overall, we found that the mini ribozyme exhibits more dynamic conformations in the presence of magnesium ions compared to calcium ions. Based on these findings, we chose to use magnesium ions in our single-molecule mechanical assays to further investigate the dynamics of hammerhead ribozymes.

This choice was motivated by the enhanced conformational dynamics observed in the MD simulations with magnesium ions, which we believed would provide a more physiologically relevant and informative context for our single-molecule studies. We have incorporated these additional findings and rationales into the revised version at line 242 and Figure S5 in the supplementary information.

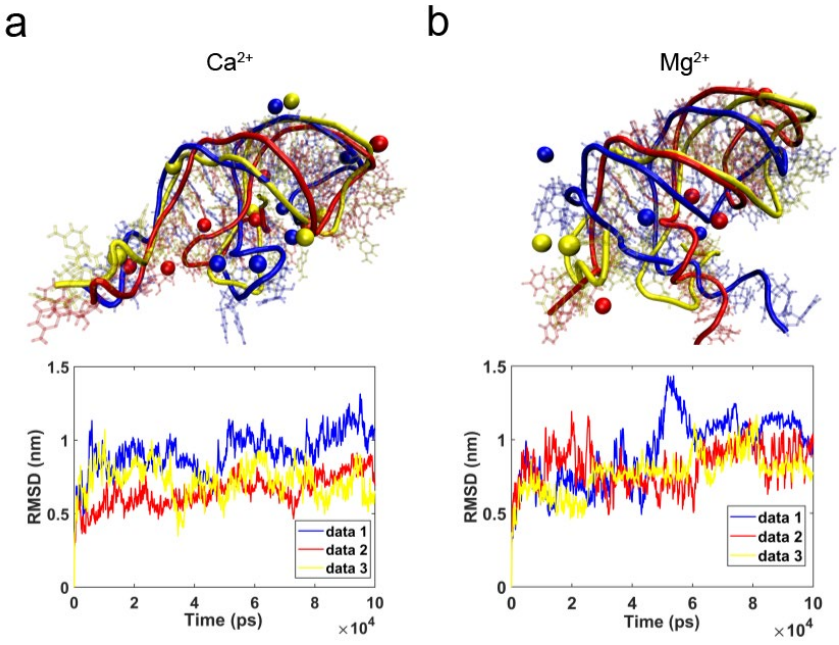

Figure S5. Molecular dynamics simulations of the mini ribozyme with divalent ions

Three data sets are presented for conditions with calcium ions (a) and magnesium ions (b). The red and yellow data sets for magnesium ions are the same as those in Figure S3b.

6. If the experiments upon adding RNA reports on the “aftermath of the cleavage reaction” (line 263) these observations are not relevant (or do not provide any information on) the mode of binding. Thus, the claim of “induced fit mechanism” does not make sense. The substrate RNA may be preferentially binding to the

alpha and/or beta state and thus shifting the equilibrium away from gamma (without “inducing” any change).

-Related to this: If the gamma state is self-inhibited and interaction with RNA results in shift in conformer distribution into the active beta state, this implies the ribozyme should become more “active” – can this be confirmed by the cleavage assay shown in the gel in Figure S2?

Response from the authors:

We appreciate the Reviewer's insightful comments. We acknowledge that we did not directly observe or measure the induced-fit process. Consequently, we have removed the statement regarding "induced-fit" in this revision and have also modified the title accordingly.

As detailed in our response to Reviewer 1's 6th question, we have developed a new single-molecule setup to directly monitor the ribozyme's digestion of the RNA substrate. We plan to report these results in the future, which should provide direct insight into the proposed induced-fit process.

Regarding the question about confirming the more active ribozyme, the results from the gel assay are collected under equilibrium conditions with a saturated RNA substrate, reflecting the fully activated ribozyme. Unlike single-molecule assays, which allow us to detect ribozyme conformations and reactions at non-equilibrium conditions and observe them individually, the gel assay provides a snapshot of the overall activity. We believe that the single-molecule experimental setup we are proposing, which can hold and monitor the RNA substrate, will enable a more detailed analysis of ribozyme activity. We will report our findings in the future.

7. It's not clear what is new with the MD simulations results, which seem to be already published. It’s also not clear how well the conformers seen in MD correlate with the experiments:

- Is the conformational distribution similar? Can you assign alpha, beta1-2 and gamma1-2 states from MD? Which conformer is the most relevant for biological activity and ribozyme-based design?

- The alpha state seems to be mostly unfolded. Can this be due to construct design or experimental conditions, or is it a common and required state for all hammerhead ribozymes?

Response from the authors:

We appreciate the Reviewer's insightful comments. We acknowledge the notable differences between the MD simulation results and the single-molecule mechanical unfolding experiments for the mini ribozyme. At this time, we are unable to definitively match the conformations observed in the single-molecule

experiments with those from the MD simulations. However, we have conducted additional MD simulations, capturing more instances of the relevant conformations. Based on our analysis, we propose that the β conformation is the most relevant for the biological activity of the ribozyme, while the γ conformation may serve as a protective state in ribozyme-based design. Regarding the α state, it is indeed mostly unfolded and is commonly observed in the unfolding pathways of the mini ribozyme. We believe this α conformation acts as a critical intermediate in various unfolding pathways, serving as a nucleation point for ribozyme folding and unfolding.

These findings and clarifications have been incorporated into Figures S3 and S5 to provide a clearer understanding of the ribozyme's conformational dynamics as inferred from our simulation data.

8. It would be helpful to include a structural (or MD) figure showing the catalysis/core and accessibility in relevant states, which is important to understand the self-inhibited and induced-fit claim by the authors.

Response from the authors:

We appreciate the Reviewer's constructive suggestion. In response, we have updated Figure 1a to include the MD structure with details of its catalytical core.

We hope these clarifications address your concerns and provide a clearer understanding of our experimental design and the implications of the observed results.

Reviewer #3:

Man Lu et al used single-molecule magnetic tweezers to explore the unfolding and folding landscapes of a hammerhead ribozyme. Their results revealed the presence of multiple RNA intermediates along the folding and unfolding pathways, shedding light on the mechanism of conformational selection and induced-fit. The mechanical conformations were further validated through MD simulations. Understanding the mechanism of conformational selection and induced-fit is crucial in the field of enzymatic catalysis, and experimental evidence plays a pivotal role in this.

This study provides quantitative experimental evidence at the single-molecule level, supporting and advancing the biological theory of conformational selection and induced-fit. Therefore, I highly recommend the publication of this study in Communications Biology, pending the resolution of the following concerns.

Response from the authors:

We sincerely appreciate the Reviewer's encouraging comments.

1. Figure 1 should show a schematic drawing of the hammerhead ribozyme that illustrates all its domains and mechanistic bonds, rather than the simple core domain and helices. This will aid readers in better understanding the ribozyme's structure.

Response from the authors:

We appreciate the Reviewer's valuable suggestion. We have updated the schematic drawing of the hammerhead ribozyme in Figure 1a to include all its domains and mechanistic bonds as recommended.

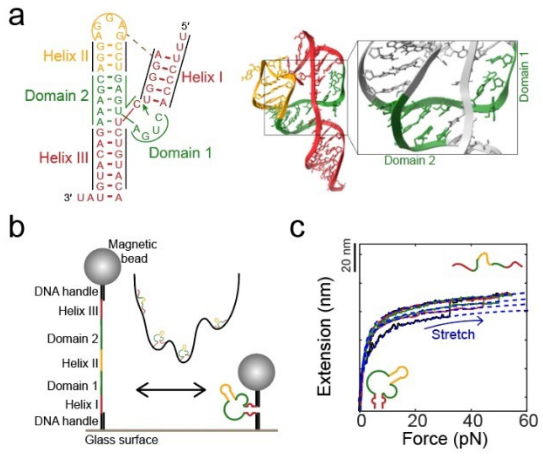

Figure 1. The structures of a hammerhead ribozyme targeting the genomic RNA sequence of SARS-CoV-2 and the experimental setup for single-molecule studies

(a) Schematic representation of the hammerhead mini ribozyme highlighting helices I-III and domains 1-2 (left), alongside the 3D structure obtained from molecular dynamics simulations (right). The green arrow marks the cleavage site.

2. In Figure 2C, the authors have identified two mechanical populations in the green panel, whereas in Figure 3C, only one population is marked. However, upon closer examination, it can be observed that the green panel of Figure 3C also exhibits two populations, albeit with a lower probability of one being omitted. It would be more equitable to discuss the fluctuation in probabilities rather than completely omitting one intermediate.

Response from the authors:

We sincerely thank the Reviewer for the thoughtful comment. If we estimate a force population using a Gaussian model, two Gaussian populations indeed provide a better fit for Figure 3C. However, it is more accurate to estimate a force population using an asymmetrical skewed distribution due to the presence of activation barriers, as theoretically suggested by OK Dudko et al. and experimentally validated by WJ Greenleaf et al. [6,7]. For this reason, we did not fit force distributions using Gaussians. Additionally, we did not apply Dudko's model due to the limited amount of data. Nonetheless, our current data is sufficient to demonstrate the number of force populations at the very least. We avoided overinterpreting the data by identifying conformations beyond clear peaks.

3. To enhance reader-friendliness, it is suggested that Figure 4b and 4d include marks indicating the disappearance of the gamma population, thus illustrating the significant conformational selection that occurs due to the ribozyme's substrate.

Response from the authors:

We sincerely thank the Reviewer for this insightful suggestion. We have marked the disappearance of the γ populations in Figures 4b and 4d.

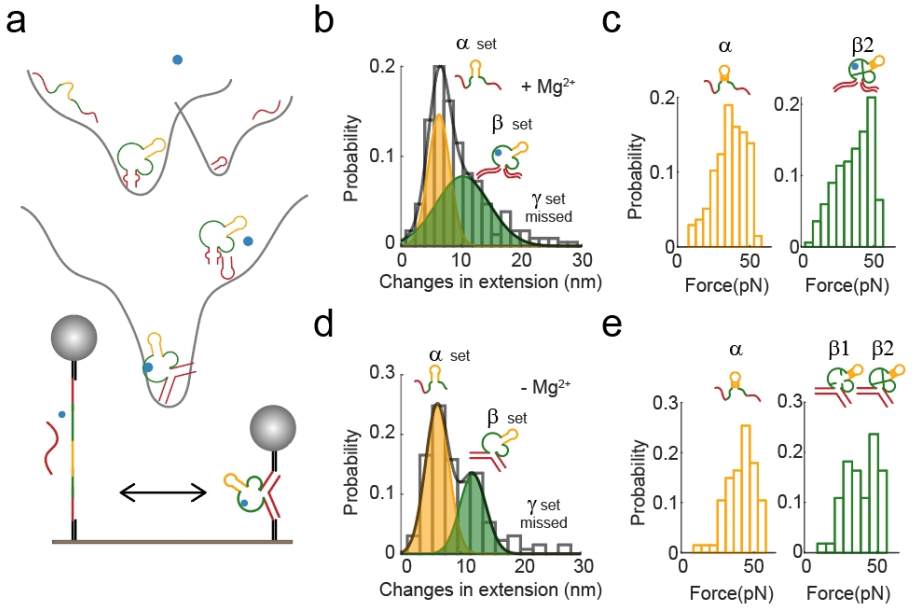

Figure 4. The induced-fit recognition between the ribozyme and SARS-CoV-2 RNA

4. To further enhance our understanding of the ribozyme's unfolding pathway, the inclusion of a series of MD snapshots would be valuable.

Response from the authors:

We appreciate the Reviewer's constructive suggestion. In response, we have updated Figure S3 and S5 to include more MD snapshots.

We hope these clarifications address your concerns and provide a clearer understanding of our experimental design and the implications of the observed results.

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